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COMPARATIVE RADIOSENSITIVITY OF PATHOGENIC BACTERIA AND VIRUSES

by

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INTRODUCTION

The radiosterilization of medical tools as it is currently
being practiced has as its principal advantage over autoclaving the
fact that it can be done without raising the temperature or even at
low temperature, and over chemical sterilization methods the fact that
it allow the material to be placed in a hermetically sealed container
prior to sterilization. It is also possible, in using high-energy
ionizing radiations (at least 1 MeV for monochromatic radiations such
as those of gamma-emitting radioactive elements, at least 3 MV of
peak tension for linear accelerators) to insure one dose of a fairly
uniform depth, without localized overdosage, even for fairly large
objects.

Nevertheless, inactivation of microbe colonies by ionizing
radiations is never an all-or-nothing operation. The sterilizing dose
always depends on the amount of microorganisms present at the outset
and on the angle of their inactivation curve. If it is decided to use
a single dose high enough to destroy all the microorganisms, one risks
radiochemical changes which, for some biological products, greatly offset
the advantages of the method. Some have also determined a "D value"
for the principal microorganisms, that is, the dose necessary for 90%
reduction of the number of live microbes (DL 90)/1/: if one understands
the nature and the extent of the contamination, one should be able
to apply a dose which is sufficient to destroy that organism, reducing
to a minimum the risk of alteration of the irradiated products; this
presupposes that the curve of microorganism inactivation is exponential
along its entire length, whatever its initial concentration something
that has rarely been established.

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In the hope of better understanding the influence of these
different factors on radiosterilization possibilities, we have deter-
mined the inactivation curve for gamma radiation of three important
pathogenic organisms: an aerobic bacterium, the tuberculosis bacillus;
an anaerobe, the agent of gaseous gangrene; and a virus, the influenza
virus. We have also studied in the case of Mycobacterium tuberculosis
and of Clostridium perfringens, the influence of microbic concentration
and length of incubation on the results; in the case of the virus,
(we have also studied) the influence of the radiation temperature.
In short, we have determined the inactivation curve of Pseudomonas,
a frequent contaminant of viral influenza cultures.

MATERIALS AND METHODS

Bacterial and viral cultures

B. C. G. (Bacillus of Calmette and Guerin) is a variety of M.
Tuberculosis, which is not pathogenic for man and thus can be handled
with a minimum of precautions; its biology and its methabolism "in
vitro" are, however, practically the same as those of virulent strains
and, in our opinion, its radiobiolpgical behavior can be considered
representative of the species. Strain 568-S 1, from the Pasteur In-
stitute in Paris, has been maintained at the Institute of Microbiology
and Hygiene of the University of Montreal since 1938, on glycerized
potato /2/. After two passages, 14 days apart, through a Sauton medium,
the microbe was grown, for the purpose of radiation, in a synthetic
liquid medium derived from the Sauton medium, prepared by one of us
(R.G.) /3/.

We have been able to establish, during the first stage of these
experiments, that the radiosensitivity of the bacillus was practically
the same if the radiation preceded or immediately followed its trans-
planting onto a cool medium. The culture is formed at $39 + 1^\circ$ C.
After incubation from 7 to 28 days the microbes of each culture dish
are separated by filtration through No 2 Whatman paper, washed with distilled
water, air-dried at 65° for three days and weighed. It has been
demonstrated that the dry weight thus obtained is an excellent criterion,
much less subjective than the bacillary count, of the viability of a
strain of the tuberculosis bacillus /4/. We express the dry weight of
the microbes as a percentage of the volume of the medium at the outset
(density: 1.014 at 20° C). When the B.C.G. vaccine has been radiated,
its viability is then tested by transplanting it from a dilution/
varying from $1/10^5$ to $1/10^7$ to a solid Dubos medium and counting the
colonies after 21 days.

At the Institute of Microbiology and Hygiene the strain SWG 121 of Cl. perfringens, type A, is kept alive in a brain medium; it is reactivated by passage through VF medium[1] and grown for the purpose of radiation in T.S.B. broth medium.[2] The optical density after incubation is measured by a Klett-Summerson photo-chronometer at 540 m.

We have utilized strains A1/AA/1/57, A2/Australia/57, B/GL 1729 and B/Taiwan/63 of the influenza virus. The virus is inocculated in 11-day old embryonic hens eggs; infected the allantoic liquid is collected after 48 hours of incubation at 35° C, placed in 10 ml sealed jars and kept at -70° C until the moment of radiation; depending on the case, the bottles are then brought back to room temperature or transfered to carbonic acid snow. By titration, the decimal dilutions of the viral suspension are inocculated into the 11-day old embryonic eggs (10 eggs per dilution); the eggs are incubated as previously, then cooled to 4° C overnight, to halt viral multiplication. The infectivity is measured by the Karber method and the haemagglutinant activity by Hirst's modified pattern test.

Conditions of radiation.

The radiations were carried out from February 1966 to March 1967 by means of a Gammacell 220 of the Atomic Energy of Canada, Ltd., charged in February 1966 with 4,700 Ci of 60 Co. Dosage was measured at that time by the ferrous sulphate method, with an accuracy of ± 2.12%.

With one exception, all the microorganisms were radiated under the conditions normally used at the Institute of Microbiology and Hygiene for the culture "in vitro". In the case of M. tuberculosis, for which there was radiation on the lyophilized B.C.G. vaccine (120 mg of wet bacilli from a 7-day culture and 10% additions of lactose were kept alive in each sealed Pyrex bottle), and on cold vaccine (120 mg--wet weight--of 14-day bacilli in 2 ml of dilute Sauton medium were placed in each 10 cc sealed bottle). In most cases, however, the tuberculosis microbe was radiated floating on the surface of the culture medium, distributed at the rate of 60 ml per culture dish, in flat-bottomed 125 cc Pyrex dishes. Cl. perfringens was radiated in suspension in 10 ml of T.S.B. broth in Hall tubes. The influenza virus, like Pseudomonas Sp., was radiated in sealed 10 cc bottles containing 10 ml of allantoic liquid. The temperature in the Gammacell radiation chamber was, at the most, 1° higher than the temperature of the room; except when carbonic acid snow was used, all the radiations were done at temperatures of 23 to 25° C.

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1. Hydrolyzed pepsic-hydrochloride of beef muscle and liver
2. Hydrolyzed trypsic casein from the Baltimore Biological Laboratories, Inc., Baltimore, Md., U.S.A.

After the moment of exposure and placement of samples in the radiation chamber, the exposure time-table varied from 450 to 325 kR (about 7,500 to 5,400 R/minute). The schedule has been calculated for each series of radiations, and the error should not have exceeded ± 7%. On the contrary, we consider it illusory to try to calculate the dosage absorbed by such small targets in such a heterogeneous environment. All the dosages hereinafter cited, therefore, are exposure dosages; we hope soon to be in a position to make direct measurements by means of LiF microdosimeters, which would permit us to express the dosage absorbed in rads.

RESULTS

Radiosensitivity of the tuberculosis bacillus

Figure 1 summarizes the results of the radiation of the cold or lyophilized B.C.G. vaccine (each point is the average of the readings made on 4 Petri dishes). These results are compatible with an exponential reduction of the viability for radiation growth doses, and it can be estimated that the DL90 ("D value") is on the order of 46 ± 8 kR for the cold vaccine and about 128 kR for the lyophilized vaccine. The difference is considerable and it should also be noted that the lyophilized vaccine received 5,300 R administered exactly like the control. If the non-radiated cold vaccine is transplanted to Petri dishes containing radiated Dubos medium, the number of colonies is the same as in the controls after an exposure of 24 kR (511 to 512 colonies per dish, on the average, at a dilution of $1:10^5$); there is a decrease in the number of colonies, not significant (471 to 507) for doses of from 49 to 390 kR but very significant (90 colonies per dish) for a medium which received 585 kR. Since the decrease in viability of the microbe is manifested after much weaker direct exposures, the change in the medium cannot then be responsible for the effects observed for dosages of less than about 400 kR.

At the time of the tests with radiated B.C.G. in its normal culture medium, we varied the "age" of the cultures at the moment of radiation and the length of incubation afterward. Normally, in the controls, transplanting onto our medium is followed by a period of latency of 2 or 3 days, then by a period of rigorous exponential growth to the tenth or eleventh day, with a generation time ("doubling time") of 24 hours. On the 12th day the development of the culture slows rather suddenly; there is then a plateau from the 14th to the 28th day, with a constant bacterial mass but an increasing proportion of dead microbes. One month after transplantation, the bacterial haze begins to "sink" in the medium and the microbes soon lose all vitality /3/.
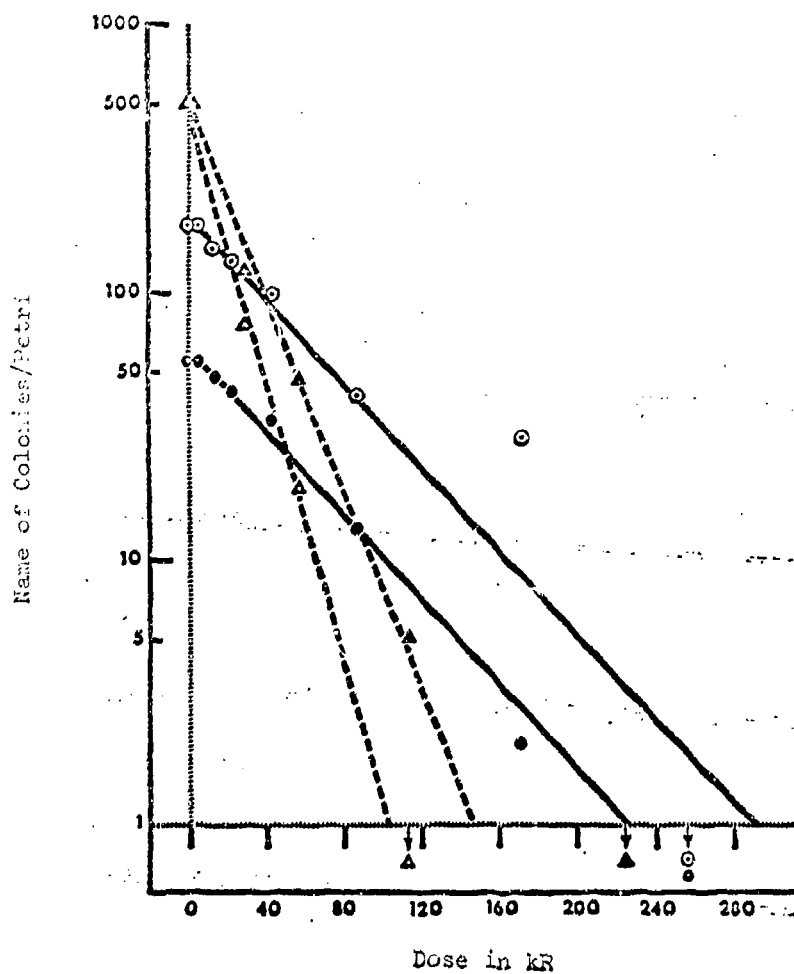
Dose in kR

FIGURE 1

Viability of irradiated B.C.G. vaccines, measured 21 days after radiation.

.... cold vaccine: △ 1:10^5 dilution
                         ▲ 1/10^6 dilution
_____ lyophilized vaccine: ⊙ number of colonies (1/10^7 dilution)
                                ● number of viable unites/cc X 10^5

Beginning with cultures two weeks old at the moment of radiation, we subjected ten cultures which came from the same number of passages of the Montreal strain, to exposures ranging from 2,500 to 110,000 R. Each series had three dishes per dose, thus each of the points in figure 2 is the average of 30 determinations. One notes, in this figure, that after 9 days of culture, that is, when the microbes are in exponential growth, there is a first-class relationship between the percentage of inhibition of development of the B.C.G. and the exposure dosage. After 14 days of culture, that relationship is still seen and in addition, the two curves are parallel, allowing a calculation of a DL90 of 29 ± 1 kR. However here too one observes, after 14 days, a bend from the beginning of the curve.

Figure 3 shows, for one particular culture, a drop in yield similar to that of the preceding test after 7 and 12 days of culture: on the other hand, if one allows the culture to develop to the 20th day, one obtains, for doses of radiation of about 30 kR, a growth at least as good as for the controls, after which the drop in yield is abrupt. This phenomenon is even more marked after 28 days of incubation, when an undeniable increase in yield is observed up to 42 kR.

Since it seemed that "radiostimulation" of the cultures was most visible when one started with old cultures and allowed them to grow the longest possible amount of time after radiation, in later tests we used cultures which were from 14 to 28 days "old" which we then incubated for 28 days, in order to be able to measure the maximum potential of the strain. Under these conditions there can be seen to be (Figure 4) a very constant increase in weight, reaching 8 per cent after one dose of 30 to 35 kR (average of 8 cultures); after this maximum there is an abrupt drop. With weak doses the increase in 14 to 28 day-old radiated cultures is practically the same; however, with doses higher than 60 kR, the "old cultures" resist appreciably higher doses than the "young" ones. The blocking of bacillar growth is complete, under the experimental conditions used, after about 110 kR for inocula of 14 days, and about 135 kR for those of 28 days. Given the shape of the curve showing the response of these bacilli to radiation, it is not possible to attribute a single value to the DL90.

Figure 5 represents the results obtained with 4 different cultures when the microbes are introduced into a medium that has just been radiated and then cultivated for 28 days. Radiation of the medium does not have any effect for doses up to 300 kR and, in two cases out of four, up to 450 kR. For the higher doses one observes a very rapid rate of yield which does not exist if the medium has received more than 700 kR. We have also reported in this figure the results obtained by radiation of the microbe in the pre-test stage. A comparison of the curves shows that, as in the case of the vaccine which is radiated and then placed on a solid medium, the phenomena observed for doses less than 300 kR can only be due to a direct effect on the microbe.
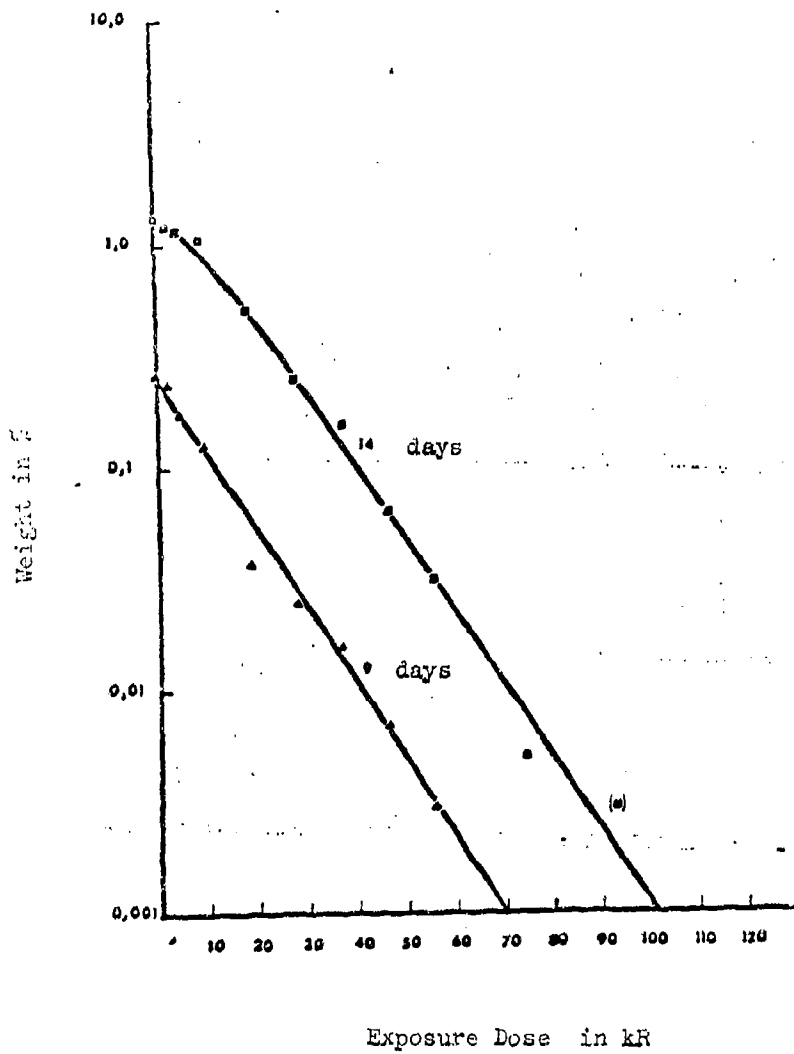
Exposure Dose   in kR

FIGURE 2

Yield of B.C.G. cultures (averages of 10 cultures) in terms
of the exposure dose by the incubation time, after radiation
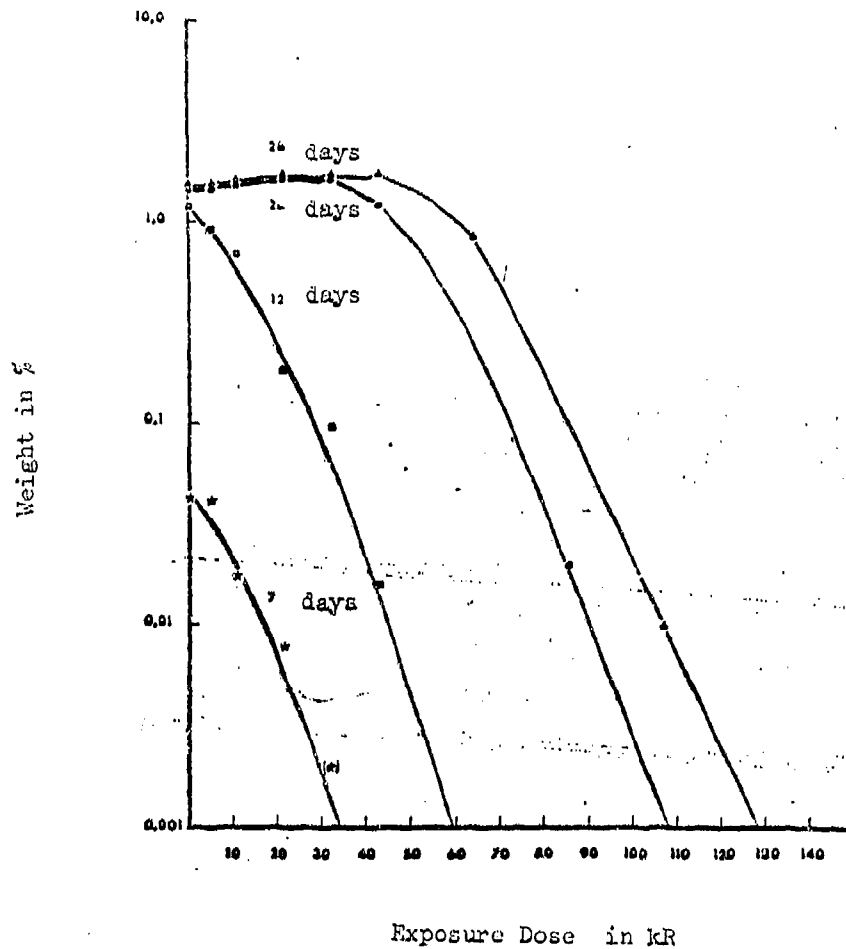from 9 to 14 days.

Exposure Dose   in kR

FIGURE 3

Yield of a typical B.C.G. culture (culture E) in terms of the
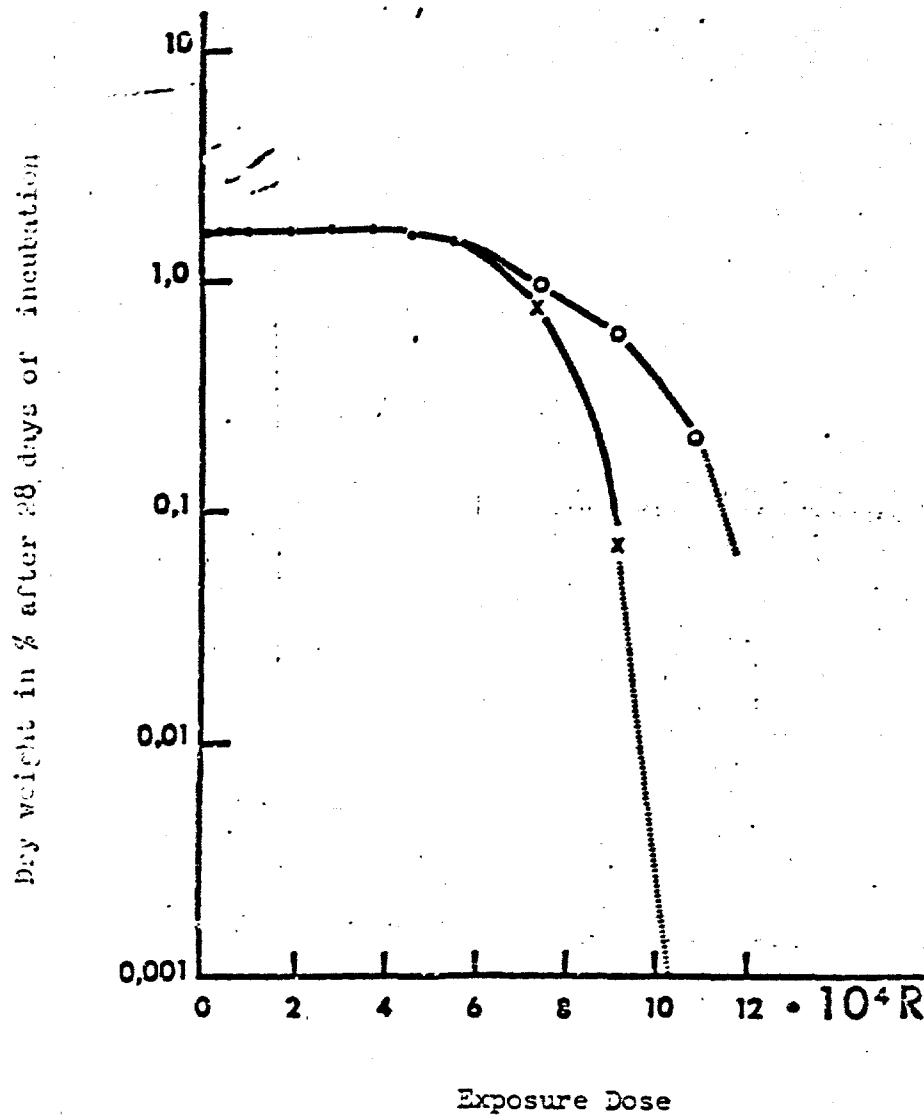exposure dose by the incubation time after irradiation of 7,
12 and 28 days.

FIGURE 4

Average yield of four B.C.G. cultured following an incubation of 28 days after irradiation, in terms of the exposure dose.

x: culture aged 14 days at the moment of radiation

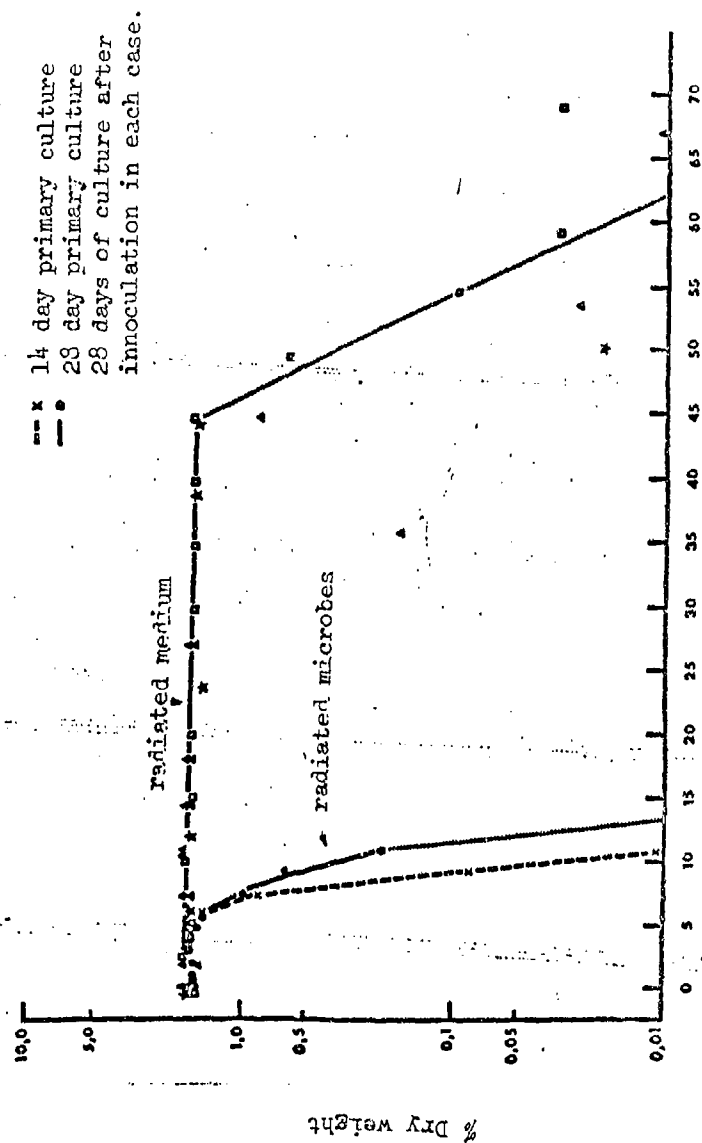o: culture aged 28 days at the moment of radiation

Dose in R x 10^4

FIGURE 5

Average yield of four cultures of B.C.G., measured following an incubation of 28 days on a medium radiated prior to transplantation, in terms of the dry weight of the microbes.

Radiosensitivity of Cl. perfringens

Figure 6 represents the variation of optic density of the cultures in terms of the exposure, so that the inoculum is 0.1 ml or 1 ml of bacillar suspension for 10 cc of T.S.B. medium for the incubation period of 1-3 days. The exposure doses used range from 5,500 R to 2,290,000 R. For each point on the curve the incubations have been repeated 3 to 10 times; each dose must be read on two columns.

After 24 hours of incubation, the inactivation curve for a weak inoculum is sigmoidal. A total inhibition of the bacterial growth is obtained for doses which are equal to or greater than 600 kR and one can calculate that for very strong doses the angle of the inactivation curve corresponds to a DL90 of about 130 kR. However, if one waits 3 days before measuring the optical density, one realizes that the figures have scarcely any significance because, in this case, at about 450 kR the inactivation curve levels off and after 1,300 kR there is another significant spurt of bacterial growth.

If the inoculum is 10 times greater, it is surprising to note that in the non-radiated controls the optical density, after 1 to 3 days, is not as high as in the first case. After 24 hours of incubation, the inactivation curve levels off after 400 kR, and the inhibition of microbial growth is only complete for doses equal to or greater than 1,900 kR. For all the reservations which we have expressed as to the value of these figures, one can estimate that, for the second part of the curve, the DL90 is in the neighborhood of 1,150 to 1,200 kR. Finally, after 72 hours the curve contains three segments with ever decreasing angles: between 1,300 and 2,300 kR. The bacterial growth is very weak but the amount does not diminish too significantly with increased exposure.

Radiosensitivity of the influenza virus

It is remarkable to note that, in all our tests, haemagglutinant power of viral suspensions has never been influenced by the strongest doses of radiation which we have used (up to 4 MR)(Figs. 7, 8 and 9). This observation is in contradiction to those of the other authors /5/ but the difference no doubt stems from technical reasons. On the contrary, the infectivity of the virus lessens in a way which at first sight seems to be exponential as a result of exposure to gamma rays; strains B are a bit more sensitive than strains A: one can calculate the following DL90's:

strain B/GL 1,739:   75 kR;   strain Al/AA/1/57:   94 kR

strain B/Taiwan/63   82 kR;   strain A2/Australia/57: 138 kR

However, it is evident, according to figure 7, that for the group Al
strain which we have used, the shape of the curve changes for the
strongest doses, supposing there are two viral populations with
different radiosensitivity, and supposing, as we have suggested in a
recent publication /6/, that there is reactivation of viral particles
(multiplicity reactivation). This phenomenon is even more pronounced
for the group A2 strain, if the virus is radiated at the sublimation
temperature of carbonic acid snow (-78.5° C) (Figure 8) and it appears
then in group B (Figure 9): the inactivation curve of strain B/Taiwan/
63, perfectly exponential at + 20° C, becomes sigmoid at -78.5° C.
Throughout this case it did not seem to us to be possible to determine
a DL90 that might be significant. The influenza virus cultures are
often contaminated by Pseudomonas. We have radiated very highly con-
taminated allantoic liquid: Figure 10 shows the radiosensitivity of
these microorganisms, compared to those of strain Al of the virus,
radiated at the same time and under the same conditions. While it took
2 MR to inactivate the virus, Pseudomonas has a DL90 of about 9 kR
and smaller exposures of 100 kR suffice to completely clear the medium.

DISCUSSIONS AND CONCLUSIONS

Comparison of the radiosensitivity of several pathogenic viruses
and bacteria requires the revision of certain classical notions which
one would have a tendency to consider valid for microorganisms in
general, whereas they only apply to particular microbes, such as
Escherichia coli.

The stimulant effect of weak doses of radiation is still very
controversial. James /7/ has shown evidence of a stimulation of mitosis
in yeast cells by weak doses of radiation: this stimulation is not
due to a mutation and is maintained for many generations. From all
evidence, the radiostimulation that we have observed in the B.C.G.
up to exposures of 40 kR is related to another phenomenon: we think
that it can be explained by selection, from a heterogeneous microbe
population, of highly prolific resistant lines. It is also probable
that the individuals destroyed by radiation furnish the resistant lines
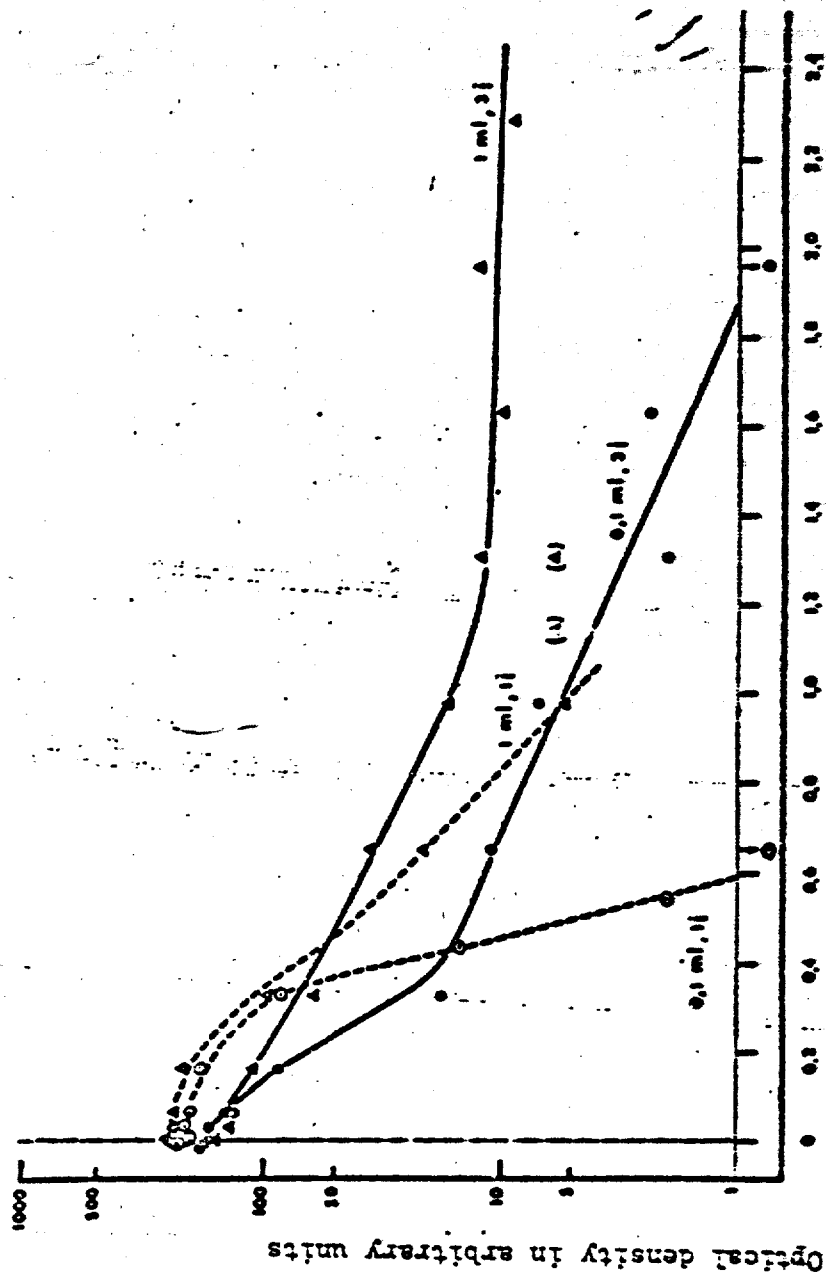with components which they can directly utilize for their synthesis.

Optical density in arbitrary units

Dose in MR

FIGURE 6

Growth of Cl. perfringens, in terms of the exposure dose.

Incubation of 1 day   ⊙ inoculum of 0.1 ml
                       ◄ inoculum of 1 ml

Incubation of 3 days   ⊙ inoculum of 0.1 ml
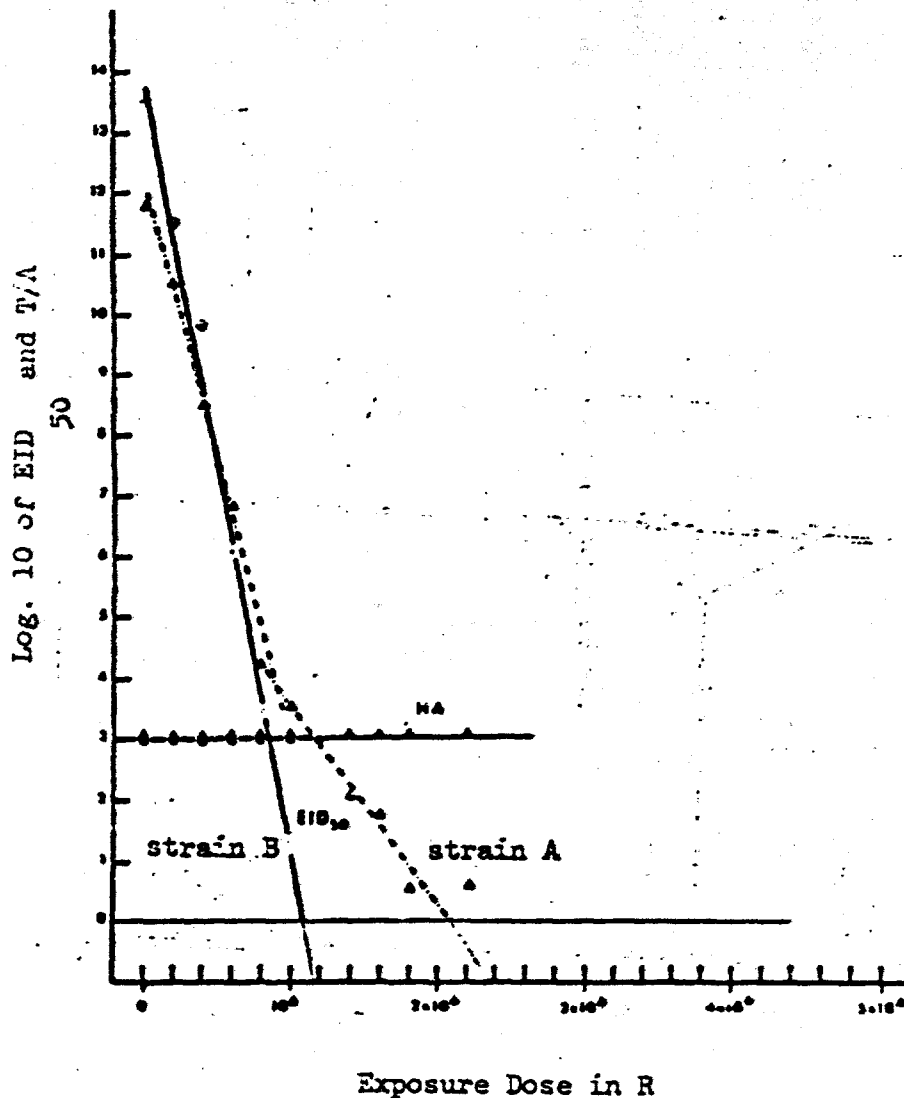   ◄ inoculum of 1 ml.

Exposure Dose in R

FIGURE 7

Comparative inactivation of strains Al/AA/1/57 and B/GL
1739 of the influenza virus, in terms of the exposure dose.
Infectivity - .-.-.-.-   strain Al/AA/1/57
                         strain B/GL 1739
(EID$_{50}$ = Egg infective dose 50%
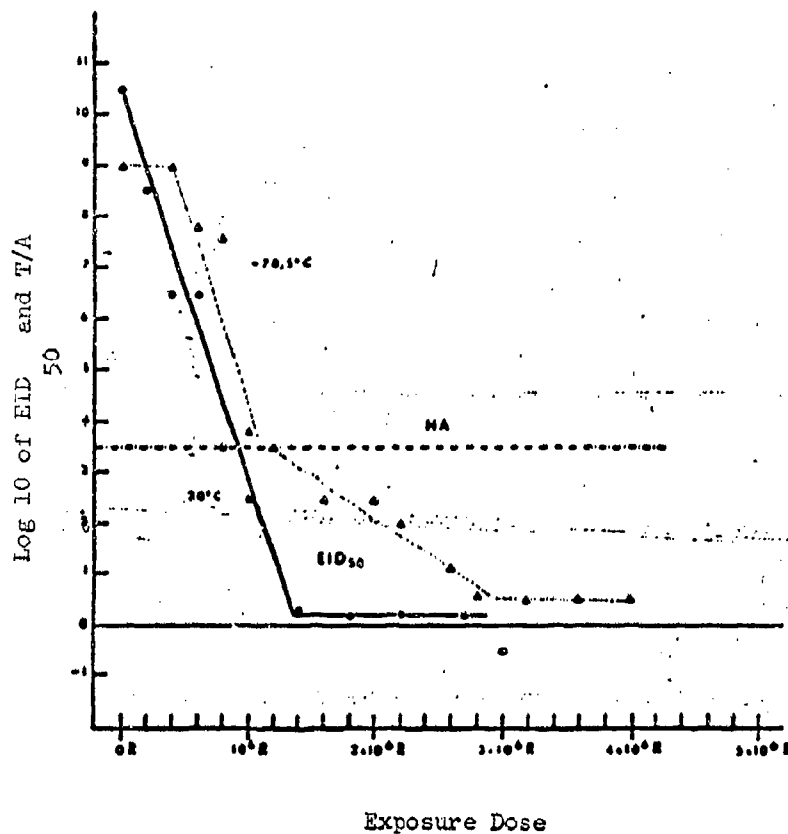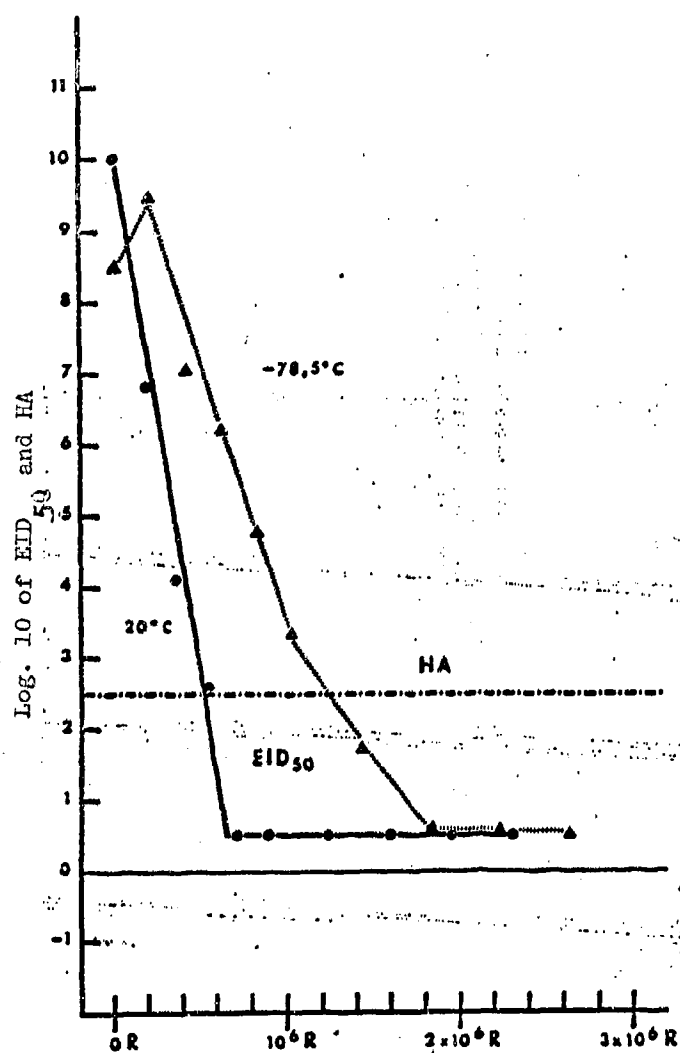
Hemagglutinant power   strain Al
                        strain B

Exposure Dose

FIGURE 8

Inactivation of strain A2/Australia/57, at + 20° C and
-78.5° C, in terms of the exposure dose (gamma rays of
^{60}Co).

Exposure Dose

FIGURE 9

Inactivation of strain B/Taiway/63, at + 20° C and
-78.5°C, in terms of the exposure dose.

The shape of the inactivation curves of Cl. perfringens seems to demonstrate that although no spores are visible under the microscope, there are extremely radioresistant spores within the cultures. After strong doses of exposure undeniable, though very limited, bacterial multiplication remains. One could suppose that, in this case, radio-chemical changes in the medium inhibit or retard the germination or growth of these radioresistant spores.

In the case of influenza virus, the peculiar shape of the inactivation curve of the virus of group A or that of the two groups at low temperature, might be explained in many ways: whatever caused the variation in the angle of the radiosensitivity curve, nothing remains to inactivate the last viral particles but an even stronger dose of radiation, the effects of the first exposures of which cannot be foreseen.

A comparitive study of our results also obliges one to discard the notion that the sensitivity of an organism is proportionate to its size and inversely proportionate to its structural complexity: Cl. perfringens -- even disregarding the spores--and under certain conditions, M. tuberculosis, are more resistant than certain strains of influenza virus. Nor do our results corroborate the idea that the radiosensitivity of a microorganism can be represented by an exponential or a simple sigmoid, corresponding to one target at a single blow or at a small number of blows /8/. Zimmer /9/ working on an apparently very simple system, an RNA solution "in vitro", has recently shown that an exponential curve can be the result of the summation of two more complex curves. We have seen above that, with Cl. perfringens, by the single fact that the initial bacillar concentration varies by a factor of 10 it completely modifies the radiosensitivity of that biological system, which is even more complex than it appeared to be at first glance. To try to formulate radiosensitivity mathematically requires too many approximations and simplifications. The DL90 (D value) notably is only significant under very precise conditions and its calculation most frequently requires that one disregard the effects of the weakest and the strongest doses: a comparison of the radiosensitivity of various organisms based on such figures thus has but little value /1/.

Two important practical conclusions seem to us to arise from this work:

--The utilization of one single dose (for example: 2.5 M rads, British Pharmacopoeia, 1963) for radiosterilization leads to an exposure which is unnecessarily high in some cases and insufficient in others. It is also necessary to know which organisms have been destroyed and what is their initial concentration. If one begins with a "bacteriologically appropriate" material, it is probable that in most cases exposure to several hundred kR will be sufficient to give a "coefficient of reduction

of viability" of the contaminants, equivalent, in practice, to steril-
ization. On the other hand there are organisms, particularly anaerobic
bacterial spores, which resist doses that are stronger than those
currently recommended: in theory, one should almost never speak of
"radiosterilization". One can also combine a relatively weak dose of
radiation with mild thermal sterilization, or simple pasteurization.
The radiation and heating conditions must be very exactly specified
in each case: Kempe, for example, showed that to reduce the number of
spores of Cl. botulinum the order in which the two operations are
carried out is important /10,11/. This combined "radio-thermal" steril-
ization may, perhaps, be the preferred method in a few years.

--"Selective radioactivation" is sometimes possible: the viability
of a microorganism used, for example, to produce a vaccine may not be
seriously affected by an exposure which completely destroys a potential
contaminant. Figure 10 shows that exposure of anti-influenza vaccine
to 100 kR or less makes it possible to halt all contamination by
Pseudomonas without significantly affecting the viability of the virus.
In another connection, the strains of the influenza vaccine that we
used are completely inactivated at the temperature of the laboratory
by exposures having from 0.8 to 2 MR, depending upon the strain, while
their hemagglutinant power is unchanged. One might expect, when giving
a dose of something like 2.5 MR, to prepare an inactive anti-influenza
vaccine, the antigenic structure of which might perhaps be more normal
than that of the inactive vaccine prepared by other methods and the
"vaccinating" power of which would thus be greater. We think that this
research direction opens a promising avenue for radiation of biological
products.
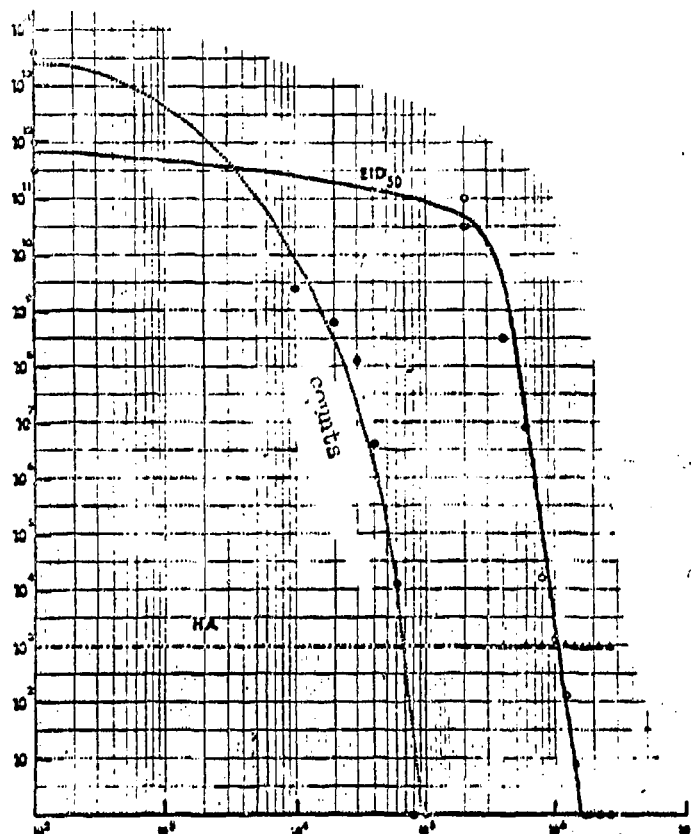
Radiation doses in Roentgens

FIGURE 10

Comparative inactivation of Pseudomonas sp. and of strain
A1/AA/1/57 of the influenza virus, in terms of the exposure
dose (^{60}Co) (N.B.: the two axes are logarithmic).

ACKNOWLEDGEMENTS

REFERENCES

/1/ WHITTET , T. D., HUGO, W.B., WILKINSON, G.R., Sterilization and disinfection, W. Heinemann, London (1965).

/2/ FRAPPIER, A., PANISSET, M., The B.C.G. Strain, Monograph of the Institute of Microbiology and Hygiene of the University of Montreal (1957).

/3/ GHYS, R., VANDERGOTEN,R., Effect of gamma rays on the tuberculosis bacillus (B.C.G. Strain), irradiated and cultivated "in vitro"., Can. J. Microbiol. (1967) in preparation.

/4/ PANISSET, M., "Physical study methods of the B.C.G.", from "Study methods of the B.C.G.", Bull. of the Intern. Union against TB. Special issue (1960) 14

/5/ POLLEY, J.R., Factors influencing inactivation of infectivity and hemagglutinin of influenza virus by gamma radiation, Can. J. Microbiol. 7(1961) 535

/6/ GILKER, J. -C., PAVILANIS, V., GHYS, R., Multiplicity reactivation in gamma irradiated influenza viruses, Nature (1967) in preparation.

/7/ JAMES, A.P., MULLER, J., Radiation induced mitotic stimulation, Radiation Research, 14 (1961)779.

/8/ HIATT, C.W., Kinetics of the inactivation of viruses, Bacter. Rev. 28 (1964) 150.

/9/ ZIMMER, K.G., Some unusual topics in radiation biology, Radiation Res. 28 (1966) 830

/10/ KEMPE, L.L., Combined effects of heat and radiation in food sterilization, Appl. Microbiol. 3 (1955) 346.

/A1/ KEMPE, L.L., "Prevention of botulism by radiation processing",
in "Botulism, proceedings of a symposium", U.S. Public Health
Service publication No 999-FP-1 (1964) 205.